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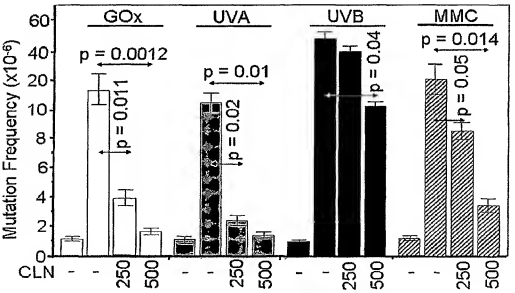

(57) Abstract: The present invention includes methods of decreasing the mutation frequency in a cell, the method including contacting cells with colostrinin, a constituent peptide thereof, an active analog thereof, or a combination thereof. Mutation frequencies decreased by the present method include spontaneous mutations and mutations induced by reactive oxygen-species (ROS), UVA radiation, UVB radiation, chemical agents and physical agents.

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USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND ANALOGS THEREOF AS ANTI-MUTAGENIC AGENTS

CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 60/834,433, filed July 31, 2006, which is incorporated by reference herein.

GOVERNMENT FUNDING

The present invention was made with government support under Grant No. ES006676, awarded by the National Institute of Environmental Health Sciences. The Government may have certain rights in this invention.

BACKGROUND

Colostrum is a component of the milk of mammals during the first few days after birth. Colostrum is a thick yellowish fluid, is the first lacteal secretion post parturition and contains a high concentration of immunogloblins (IgG, lgM, and IgA) and a variety of proteins with unknown functions. Colostrum also contains various cells such as granular and stromal cells, neutrophils, monocyte/ macrophages, and lymphocytes and includes growth factors, hormones, and cytokines. Unlike mature breast milk, colostrum contains low sugar, low iron, but is rich is lipids, proteins, mineral salts, vitamins, and immunoglobins.

Colostrum also includes a proline-rich polypeptide aggregate or complex, which is referred to as colostrinin (CLN). One peptide fragment of colostrinin is Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (SEQ ID NO:31). Colostrinin and this fragment have been identified as useful in the treatment of disorders of the central nervous system, neurological disorders, mental disorders, dementia, neurodegenerative diseases, Alzheimer's disease, motor neuron disease, psychosis, neurosis, chronic disorders of the immune system, diseases with a bacterial and viral etiology, and acquired immunological deficiencies, as set forth in International Publication No. WO 98/14473.

Although certain uses for colostrinin have been identified, it would represent advancement in the art to discover and disclose other uses for colostrinin and constituent peptides thereof.

SUMMARY OF THE INVENTION

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, under conditions effective to accomplish at least one of the following: decrease the frequency of reactive oxygen-species (ROS) induced mutations; decrease the frequency of mutations induced by chemical agents; or decrease the frequency of mutations induced by physical agents.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by chemical agents and/or physical agents and is not induced by reactive oxygen-species (ROS).

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced not only by reactive oxygen-species (ROS), but is also induced also by chemical agents and/or physical agents.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the

mutation frequency is induced by a chemical agent and/or a physical agent and is not induced by UVA or UVB radiation.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is UVA radiation-induced mutagenesis.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is UVB radiation-induced mutagenesis.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by a chemical agent.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by a physical agent.

The present invention includes a method of decreasing spontaneous mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof.

The present invention includes a method of decreasing mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, wherein the mutation frequency is environmentally induced.

The present invention includes a method of decreasing the mutation frequency in a cell, the method including contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active

analog of a constituent peptide of colostrinin, and combinations thereof; wherein the modulator is administered in conjunction with one or more therapeutic agents that induce mutations in genomic and/or mitochondrial DNA. In some embodiments, the therapeutic agent is chemotherapy or radiation therapy for cancer.

In some embodiments of the methods of the present invention, the modulator is a constituent peptide of colostrinin or an analog thereof. In some embodiments of the methods of the present invention, the constituent peptide of colostrinin is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLQPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), VESYVPLFP (SEQ ID NO:31), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34), RPKHPIKHQGLPQEVLNENLLRF (SEQ ID NO:35), FVAPFPEVFGKEKV (SEQ ID NO:36), SDIPNPIGSENSEKTTMPLW (SEQ ID NO:37), GPVRGPFPI (SEQ ID NO:38), EPVLGPVRGPFPI (SEQ ID NO:39), VPYPQRDMPIQ (SEQ ID NO:40), SLSQSKVLPVPQKAVPYPQRDMPIQ (SEQ ID NO:41), RPKHPIKHQ (SEQ ID NO:42); EPVLGPVR (SEQ ID NO:43); RGPFPIIV (SEQ ID NO:44); PPPPLLPSRPLPPLPPP (SEQ ID NO:45); PPPPPPPPP (SEQ ID NO:46); and combinations thereof.

In some embodiments of the methods of the present invention an active analog of a constituent peptide of colostrinin comprises a peptide having an amino acid sequence with at least about 15 percent proline and having at least about 70 percent structural similarity to one or more constituent peptides of colostrinin.

In some embodiments of the methods of the present invention the cell is present in a cell culture, a tissue, an organ, or an organism. In some embodiments of the methods of the present invention the cell is a mammalian cell. In some embodiments of the methods of the present invention the cell is a human cell.

The present invention includes the use of a compound selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof in the manufacture of a medicament for reducing the mutation frequency in a cell.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B establish that colostrinin (CLN) has no mutagenic potential. Fig. 1A shows CLN decreases spontaneous mutation frequency. Parallel cultures of V79 cells were treated with increasing concentrations of CLN for 48 hours (3.6 population doublings (PDs)) in growth medium. Then cells were placed in 10-cm Petri dishes (10^6 cells per dish) in six parallel experiments and incubated further in medium containing 6-Thioguanine (TG) (7 μg per ml). Seven days later cell colonies were fixed, stained with crystal violet and counted. Fig. 1B shows long-term treatment with CLN exerts no mutagenic potential. Cells were maintained with or without CLN (concentrations are as indicated) for ~40 PDs and mutation frequencies determined. As a control, cells were treated with 20 ng per ml GOx. In Figs. 1A and 1B, mutation frequencies were calculated as described previously (Bradley et al., 1981, *Mutat Res*, 87-81-142; Albrecht et al., 1997, *Virology*, 230:48-61).

Figures 2A to 2C demonstrate that CLN decreases frequency of ROS-induced 6-TG resistant cells. Fig. 2A shows the effect of CLN on ROS-induced mutation frequency. Cells were grown in the presence of CLN for 24 hours and then treated with GOx (20 ng per ml) for one hour (at 37°C), washed and further incubated for 1 day with CLN. Fig. 2B shows the effect of CLN pre-treatment on MF induced by ROS. Cells were grown for 24 hours in medium containing CLN, and GOx-treated as above, washed and further incubated for one day without CLN. Fig. 2C shows the effect of post-treatment with CLN on ROS-induced mutation frequency. Cells were grown without CLN, GOx-treated as in Fig. 2A, washed, and incubated further for an additional 24 hours in the presence of CLN. In Figs. 2A-2C, cells were trypsinized, counted and seeded in 6-TG containing medium (10^6

cells per 10 cm dish). Seven days later colonies were stained, and counted and mutation frequencies were calculated (Bradley et al., 1981, *Mutat Res*, 87-81-142; Albrecht et al., 1997, *Virology*, 230:48-61).

Figures 3A and 3B show the effect of CLN on UVA- and UVB-induced mutation frequency. Fig. 3A shows the frequency of *hprt* mutations in UVA-irradiated cells ±CLN. Cells were grown ±CLN for 24 hours and at 50% confluence irradiated with UVA (87 kJ per m^2), and CLN-containing media were added back. At 24 hours later cells were placed (10^6 cells per dish) in 6-TG-containing selection medium. Fig. 3B shows CLN exerts protection against UVB-induced mutations. Experiments were carried out as in Fig. 3A, but cells were irradiated with UVB (4.1 kJ per m^2). In Figs. 3A and 3B, mutation frequencies were calculated as in the legend to Fig. 1.

Figures 4A and 4B show the effect of CLN on frequency of 6-TG-resistant mutants in alkylating agent-treated cell cultures. Fig. 4A shows the change in MF by CLN in MMS-treated cells. Parallel cell cultures (~30% confluence) were treated with 100, 250 or 500 ng/ml CLN and 24hours later MMS (LD_{10} = 115 μM) pulsed for one hour. Excess MMS was removed by washing and the same amount of CLN was added to the corresponding cultures for one day. Fig. 4B shows decreased MF by CLN in MMC-treated cells. Experiments were undertaken as above in Fig. 4A, but cells were MMC-treated. In Figs. 4A and 4B, mutation frequencies were determined as in the legend to Fig. 1.

Figure 5 shows that CLN decreases mutation frequency in human cells at the hprt locus. Human airway epithelial cell cultures at 50% confluence were treated with CLN (250, 500 ng per ml) for 24 hours and then exposed to GOx (110 ng per ml for one hour); UVA (105 kJ per m^2), UVB (7.1 kJ per m^2) or MMC (53 μM for one hour). Cells were washed and growth medium containing CLN was added to the corresponding cultures for 52 h (two PDs). Mutation frequencies were determined as in the legend to Fig. 1.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

Colostrinin (CLN), a complex of proline-rich polypeptides derived from colostrum, induces various signaling pathways common to cell proliferation and

differentiation, and mediates events similar to those of hormones and neurotrophins, leading to neurite outgrowth (see, for example, Bacsi et al. *Cell Mol Neurobiol* 25: 1123-1139, 2005; U.S. Patent No. 6,852,685; and WO 02/13852). CLN is an important immune-modulator; it has been shown to induce various cytokines, mediate maturation and differentiation of murine thymocytes (see, for example, Janusz et al. *Arch Immunol Ther Exp (Warsz)* 41: 275-279, 1993; Zimecki et al. *Arch Immunol Ther Exp (Warsz)* 32: 191-196, 1984; Zimecki et al. *Arch Immunol Ther Exp (Warsz)* 32: 197-201, 1984; U.S. Patent No. 6,903,068; and WO 02/13849) and promote peripheral blood leukocyte proliferation (see, for example, Janusz et al. *Arch Immunol Ther Exp (Warsz)* 41: 275-279, 1993; and Stanton et al. *Psychogeriat. Annuals* 4: 67-75, 2001). CLN decreases the intracellular oxidative stress levels, and reduces 4-hydroxynonenal (4HNE)-mediated cellular damage and cellular signaling in cultured cells (see, for example, Boldogh et al. *Psychogeriat. Annuals* 4: 57-65, 2001, Boldogh et al. *J Mol Neurosci* 20: 125-134, 2003; U.S. Patent Nos 6,500,798 and 6,939,847; WO 02/13850; and U.S. Patent Application Nos. 2005/0042300 and 2004/0266681). CLN protects neuroblastoma cells from beta amyloid-induced apoptosis by inhibiting amyloid aggregation (Schuster et al. *Neuropeptides* 39: 419-426, 2005). Most importantly, CLN administration to Alzheimer's patients resulted in stabilization of cognitive functions and improvement in the ability to perform routine domestic functions (see, for example, Bilikiewicz et al. *J Alzheimers Dis* 6: 17-26, 2004, Leszek et al. *Arch Immunol Ther Exp (Warsz)* 47: 377-385, 1999). Moreover, CLN administration to one-day-old domestic chicks significantly enhanced long-term memory retention in a passive avoidance model (Stewart and Banks. *Neurobiol Learn Mem* 86: 66-71, 2006).

With the present invention, it has been discovered that colostrinin, constituent peptides of colostrinin, and analogs of constituent peptides can be used as modulators of mutation frequencies (MF). More specifically, colostrinin, constituent peptides of colostrinin, and analogs of constituent peptides modulate mutation frequency by decreasing or reducing mutation frequency. A mutation is a permanent alteration in the sequence of nitrogenous bases of a DNA molecule. The result of a mutation may be a change in the end-product specified by that gene. In some cases, a mutation can be beneficial if a new metabolic activity arises in a microorganism, or it can be detrimental if a metabolic activity is altered or lost. Mutation frequencies may be determined by any of many known methods,

including, but not limited to, any of those described herein. Mutations may include somatic mutations and/or germline mutations. Somatic mutations occur in a somatic cell, for example, the bone marrow or liver, may make the cell cancerous, and/or may kill the cell. Whatever the effect, the ultimate fate of a somatic mutation is to disappear when the cell in which it is present dies. Germline mutations, in contrast, may be found in every cell descended from the zygote in which the mutant gamete contributed. Mutations may include genomic and/or mitochondrial mutations. Mutations may include spontaneous mutations, environmentally induced mutations and/or mutations induced by the administration of another agent, for example, the administration of a therapeutic agent for the treatment of a disease, such as, for example, a chemotherapeutic agent or radiation.

The present invention provides methods of decreasing the mutation frequency in a cell by contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof.

With the methods of the present invention, a cell may be contacted with a modulator under conditions effective to accomplish one or more of the following: decrease the frequency of reactive oxygen-species (ROS) induced mutations; decrease the frequency of mutations induced by chemical agents; or decrease the frequency of mutations induced by physical agents. For example, the present invention includes methods of decreasing the frequency of mutations induced by reactive oxygen-species, chemical agents, and physical agents; methods of decreasing the frequency of mutations induced by reactive oxygen-species and chemical agents; methods of decreasing the frequency of mutations induced by reactive oxygen-species and physical agents; methods of decreasing the frequency of mutations induced by chemical agents and physical agents; methods of decreasing the frequency of mutations induced by reactive oxygen-species; methods of decreasing the frequency of mutations induced by chemical agents; and methods of decreasing the frequency of mutations induced by physical agents. The present invention also provides methods of decreasing the mutation frequency in a cell wherein the mutation frequency is induced by chemical agents and/or physical agents and is not induced by reactive oxygen-species (ROS).

Reactive oxygen species (ROS) or free radicals are chemical species that have a single unpaired electron in an outer orbit. They are highly reactive chemical

radicals that are generated as products of oxygen degradation. Reactive oxygen species include, for example, oxygen ions, free radicals and both inorganic and organic peroxides. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROSs form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. Reactive oxygen species are potent oxidants that can attack chromosomal DNA and free nucleotides, leading to oxidative DNA damage that causes genetic alterations.

With the methods of the present invention, a cell may be contacted with a modulator under conditions effective to accomplish one or more of the following: decrease the frequency of UVA radiation-induced mutations; decrease the frequency of UVB radiation-induced mutations; decrease the frequency of mutations induced by chemical agents; and/or decrease the frequency of mutations induced by physical agents. For example, the present invention includes methods of decreasing the frequency of mutations induced by UVA irradiation, UVB irradiation, chemical agents, and physical agents; methods of decreasing the frequency of mutations induced by UVA irradiation, chemical agents and physical agents; methods of decreasing the frequency of mutations induced by UVB irradiation, chemical agents and physical agents; methods of decreasing the frequency of mutations induced by UVB irradiation; methods of decreasing the frequency of mutations induced by UVA irradiation; methods of decreasing the frequency of mutations induced by chemical agents and physical agents; methods of decreasing the frequency of mutations induced by chemical agents; and methods of decreasing the frequency of mutations induced by physical agents.

The present invention also provides methods of decreasing the mutation frequency wherein the mutation frequency is not induced by UVB irradiation; methods of decreasing the mutation frequency wherein the mutation frequency is not induced by UVA irradiation; and methods of decreasing the mutation frequency wherein the mutation frequency is not induced by UVA irradiation or UVB irradiation.

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than soft X-rays. The sun emits ultraviolet

radiation in the UVA, UVB, and UVC bands, but because of absorption in the atmosphere's ozone layer, 99% of the ultraviolet radiation that reaches the Earth's surface is UVA. Ultraviolet (UV) irradiation present in sunlight is an environmental human carcinogen and the toxic effects of UV from natural sunlight and therapeutic artificial lamps are a major concern for human health. As used herein, UVA radiation is that portion of the electromagnetic spectrum from 400 to 320 nanometers (nm). As used herein, UVB radiation is that portion of the electromagnetic spectrum from 290 to 320 nm.

A wide variety of chemical agents that induce genetic mutations are known in the art, including, for example, base analogs (such as, for example, 5-bromouracil), acridines (such as, for example, 2,8,-diamine acridine, also known as proflavin), alkylating agents (such as, for example, N-ethyl-N-nitrosourea (ENU) and ethyl methanesulfonate (EMS)), DNA intercalating agents (such as, for example, ethidium bromide), and DNA cross linkers (such as, for example, mitomycin C (MMC) and platinum).

The present invention also provides methods of decreasing spontaneous mutation frequencies and environmentally induced mutations frequencies. With the present invention, cells may be contacted with one or more modulators *in vitro* or and *in vivo*.

With the present invention, colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, or combinations thereof, may be used to reduce the frequency of mutations induced by any combination of one or more of the various mechanisms by which genetic mutations are induced; for example, reduce the frequency of mutations induced by reactive oxygen species (ROS), exposure to chemical agents, and exposure to physical agents; reduce the frequency of mutations induced by chemical agents and/or physical agents; reduce the frequency of mutations induced by reactive oxygen species (ROS); reduce the frequency of mutations induced by chemical agents; reduce the frequency of mutations induced by physical agents; reduce the frequency of mutations induced by chemical agents and/or physical agent but not the frequency of mutations induced by reactive oxygen species (ROS); reduce the frequency of mutations induced by reactive oxygen species (ROS) in addition to reducing the frequency of mutations induced by chemical agents and/or physical agents; reduce the frequency of mutations induced by a UVA-based mechanism; reduce the frequency of mutations

induced by a UVB-based mechanism; or reduce the frequency of mutations induced by both UVA-based and UVB-based mechanisms.

Colostrinin (CLN) is a uniform, low-molecular weight, proline-rich mix of peptides purified from colostrum via various chromatographic steps, including ion exchange, affinity and molecular sieving, (see, for example, Janusz et al. *Arch Immunol Ther Exp (Warsz)* 41: 275-279, 1993; Janusz et al. *FEBS Lett* 49: 276-279, 1974; and WO 98/14473) or using a two-step extraction/purification method that includes methanol extraction and ammonium sulfate precipitation as the general principles (Kruzel et al. *Protein J*, 23:127-133, 2004 and WO 2004/081038). When compared with the original material, CLN shows a characteristic pattern of peptides in SDS PAGE and an amino acid analysis characterized by high content of proline (22%), a high proportion of nonpolar amino acids, a low percentage of glycine, alanine, arginine, histidine, and no tryptophan, methionine, and cysteine residues, a similar pattern of HPLC profiles, and the ability to induce IFN gamma and TNF alpha. CLN is composed of peptides, the aggregate of which has a molecular weight range between about 5.8 to about 26 kilodaltons (kDa) determined by polyacrylamide gel electrophoresis. It has a greater concentration of proline than any other amino acid. CLN may also be referred to as "colostrinin complex" "proline rich polypeptide, "PRP," "proline-rich polypeptide aggregate," "proline-rich polypeptide aggregate derived from colostrum," a "proline-rich polypeptide complex," a "proline-rich polypeptide complex derived from colostrum," and "neuroprotective proline-rich polypeptides." Ovine colostrinin has been found to have a molecular weight of about 18 kDa (Kruzel et al., *J. Mol. Neurosci.* 17:379-89, 2001) and includes three non-covalently linked subunits having a molecular weight of about 6 kDa and has about 22% proline by weight.

For certain embodiments, the anti-mutagenic agent is a constituent peptide of colostrinin. Constituent peptides of clostrinin include a number of peptides ranging from about 3 amino acids to about 22 amino acids or more. These can be obtained by various known techniques, including isolation and purification involving electrophoresis or synthetic techniques. Methods of obtaining colostrinin, include, but are not limited to, those described, for example, in WO 98/14473, WO 04/081038, US Patent Application 2005/0085422, and US Patent Application 2006/0154871. Using HPLC and Edman degradation, over 60 constituent peptides of colostrinin have been identified, which can be classified into several groups: (A)

those of unknown precursor; (B) those having a β-casein homologue precursor; (C) those having a β-casein precursor; and (D) those having an annexin precursor (WO 2004/081038). These peptides are described in International Patent Publication No. WO 00/75173 and can be synthesized according to well-known synthetic methods. These constituent peptides of colostrinin can be derived from colostrinin or chemically synthesized, include, but are not limited to, MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIPN (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ (SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP (SEQ ID NO:24); QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26); LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); VESYVPLFP (SEQ ID NO:31); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and MHQPPQPLPPTVMFP (SEQ ID NO:34). These can be classified as follows: (A) those of unknown precursor include SEQ ID NOs:2, 6, 7, 8, 10, 11, 14, and 33; (B) those having a β-casein homologue precursor include SEQ ID NOs:1, 3, 4, 5, 9, 12, 13, 15, 16, 17, and 31; (C) those having a β-casein precursor include SEQ ID NOs:18 (casein amino acids 74-83), 19 (casein amino acids 84-92), 20 (casein amino acids 93-102), 21 (casein amino acids 103-120), 22 (casein amino acids 121-138), 23 (casein amino acids 139-156), 24 (casein amino acids 157-163), 25 (casein amino acids 164-173), 26 (casein amino acids 174-179), 27 (casein amino acids 180-201), 28 (casein amino acids 202-208), 29 (casein amino acids 214-222), 32 (casein amino acids 203-214), and 34 (casein amino acids 159-173); and (D) those having an annexin precursor include SEQ ID NO:30 (annexin amino acids 203-220).

Additional constituent peptides of colostrinin include the following peptides with sequence homology to casein: casein alpha S-1 derived peptide 16-38

RPKHPIKHQGLPQEVLNENLLRF (SEQ ID NO:35), casein alpha S-1 derived peptide 39-52 FVAPFPEVFGKEKV (SEQ ID NO:36), casein alpha S-1 derived peptide 195-214 SDIPNPIGSENSEKTTMPLW (SEQ ID NO:37), casein beta derived peptide 199-207 GPVRGPFPI (SEQ ID NO:38), casein beta derived peptide 195-207 EPVLGPVRGPFPI (SEQ ID NO:39), casein beta derived peptide 199-188 VPYPQRDMPIQ (SEQ ID NO:40), casein beta derived peptide 164-188 SLSQSKVLPVPQKAVPYPQRDMPIQ (SEQ ID NO:41); peptide RPKHPIKHQ (SEQ ID NO:42); peptide having an N-terminal sequence of RPKHPIKHQ (SEQ ID NO:42); casein-derived EPVLGPVR (SEQ ID NO:43); casein-derived RGPFPIIV (SEQ ID NO:44); Fas Ligand-derived PPPPLLPSRPLPPLPPP (SEQ ID NO:45); and Fas Ligand-derived PPPPPPPPP (SEQ ID NO:46). Further colostrinin constituent peptides include any of those described in WO 2004/081038, WO 00/75173, WO 02/46211, US Patent Application 2005/0085422, and US Patent Application 2006/0154871.

In some embodiments, a preferred group of constituent peptides includes: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); and combinations thereof.

The polypeptides of SEQ ID NOs:1-46 and other constituent peptides of colostrinin can be in their free acid form or they can be amidated at the C-terminal carboxylate group. The present invention also includes analogs of the polypeptides of SEQ ID NOs:1-46, which includes polypeptides having structural similarity with SEQ ID NOs:1-46. An "analog" of a constituent peptide of colostrinin includes at least a portion of the colostrinin peptide, wherein the portion contains deletions or additions of one, two, three, one to two, one to three, or more contiguous or noncontiguous amino acids, or containing one, two, three, one to two, one to three, or more amino acid substitutions. An "analog" can thus include one, two, three, one to two, one to three, or more additional amino acids at one or both of the N or C terminii of the constituent peptides described herein. Substitutes for an amino acid in the polypeptides of the invention are preferably conservative substitutions, which are selected from other members of the class to which the amino acid belongs. For example, it is well-known in the art of protein biochemistry that an amino acid

belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can generally be substituted for another amino acid without substantially altering the structure of a polypeptide.

For the purposes of this invention, conservative amino acid substitutions are defined to result from exchange of amino acids residues from within one of the following classes of residues: Class I: Ala, Gly, Ser, Thr, and Pro (representing small aliphatic side chains and hydroxyl group side chains); Class II: Cys, Ser, Thr and Tyr (representing side chains including an -OH or -SH group); Class III: Glu, Asp, Asn and Gln (carboxyl group containing side chains): Class IV: His, Arg and Lys (representing basic side chains); Class V: Ile, Val, Leu, Phe and Met (representing hydrophobic side chains); and Class VI: Phe, Trp, Tyr and His (representing aromatic side chains). The classes also include related amino acids such as 3Hyp and 4Hyp in Class I; homocysteine in Class II; 2-aminoadipic acid, 2-aminopimelic acid, (γ-carboxyglutamic acid, β-carboxyaspartic acid, and the corresponding amino acid amides in Class III; ornithine, homoarginine, N-methyl lysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, homoarginine, sarcosine and hydroxylysine in Class IV; substituted phenylalanines, norleucine, norvaline, 2-aminooctanoic acid, 2-aminoheptanoic acid, statine and β-valine in Class V; and naphthylalanines, substituted phenylalanines, tetrahydroisoquinoline-3-carboxylic acid, and halogenated tyrosines in Class VI.

Preferably, the active analogs of a constituent peptide include polypeptides having a relatively large number of proline residues. Because proline is not a common amino acid, a "large number" preferably means that a polypeptide includes at least about 15% proline (by number), and more preferably at least about 20% proline (by number). Most preferably, active analogs include more proline residues than any other amino acid.

Analogs of constituent peptides of colostrinin include peptides having structural similarity. Structural similarity is generally determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. Preferably, two amino acid sequences are compared using the

Blastp program, version 2.0.9, of the BLAST 2 search algorithm, available on the world wide web at ncbi.nlm.nih.gov/gorf/bl2.html. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identity." Preferably, an active analog of a constituent peptide of colostrinin has a structural similarity to colostrinin or one or more of its constituent peptides (preferably, one of SEQ ID NOs:1-46) of at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 97% identity, at least about 98% identity, or at least about 99% identity.

Colostrinin or any combination of its constituent peptide thereof or active analogs thereof can be derived (preferably, isolated and purified) naturally such as by extraction from colostrum or can be synthetically constructed using known peptide polymerization techniques. For example, the peptides of the invention may be synthesized by the solid phase method using standard methods based on either t-butyloxycarbonyl (BOC) or 9-fluorenylmethoxy-carbonyl (FMOC) protecting groups. This methodology is described by G.B. Fields et al. in Synthetic Peptides: A User's Guide, W.M. Freeman & Company, New York, NY, pp. 77-183 (1992). Moreover, gene sequence encoding the colostrinin peptides or analogs thereof can be constructed by known techniques such as expression vectors or plasmids and transfected into suitable microorganisms that will express the DNA sequences thus preparing the peptide for later extraction from the medium in which the microorganism are grown. For example, U.S. Patent No. 5,595,887 describes methods of forming a variety of relatively small peptides through expression of a recombinant gene construct coding for a fusion protein which includes a binding protein and one or more copies of the desired target peptide. After expression, the fusion protein is isolated and cleaved using chemical and/or enzymatic methods to produce the desired target peptide.

The peptides used in the methods of the present invention may be employed in a monovalent state (i.e., free peptide or a single peptide fragment coupled to a carrier molecule). The peptides may also be employed as conjugates having more than one (same or different) peptide fragment bound to a single carrier molecule. The carrier may be a biological carrier molecule (e.g., a glycosaminoglycan, a

proteoglycan, albumin or the like) or a synthetic polymer (e.g., a polyalkyleneglycol or a synthetic chromatography support). Typically, ovalbumin, human serum albumin, other proteins, polyethylene glycol, or the like are employed as the carrier. Such modifications may increase the apparent affinity and/or change the stability of a peptide. The number of peptide fragments associated with or bound to each carrier can vary, but from about 4 to 8 peptides per carrier molecule are typically obtained under standard coupling conditions.

For instance, peptide/carrier molecule conjugates may be prepared by treating a mixture of peptides and carrier molecules with a coupling agent, such as a carbodiimide. The coupling agent may activate a carboxyl group on either the peptide or the carrier molecule so that the carboxyl group can react with a nucleophile (e.g., an amino or hydroxyl group) on the other member of the peptide/carrier molecule, resulting in the covalent linkage of the peptide and the carrier molecule. For example, conjugates of a peptide coupled to ovalbumin may be prepared by dissolving equal amounts of lyophilized peptide and ovalbumin in a small volume of water. In a second tube, 1-ethyl-3-(3-dimethylamino-propyl)-carboiimide hydrochloride (EDC; ten times the amount of peptide) is dissolved in a small amount of water. The EDC solution was added to the peptide/ovalbumin mixture and allowed to react for a number of hours. The mixture may then dialyzed (e.g., into phosphate buffered saline) to obtain a purified solution of peptide/ovalbumin conjugate. Peptide/carrier molecule conjugates prepared by this method may contain about 4 to 5 peptides per ovalbumin molecule.

The present methods of reducing mutation frequency have wide applicability. Colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be applied in any application in which an inhibitor of mutagenesis and/or carcinogenesis is desired. For example, colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be applied to cell or administered to a subject to prevent, inhibit, and/or reduce the effects of natural and/or man-made environmental mutagens on the cell or subject. Colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be administered along with additional agents demonstrating anti-mutagenic effects, such as for example, vitamins, thiol compounds, porphyrin derivatives, and polyphenols, the antigenotoxicity of which

are well established in various genetic tests. Such additional agents may or may not act synergistically with colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, or combinations thereof.

Colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be administered as an adjunct therapy to the administration of an antibiotic for the treatment of a bacterial infection, reducing the mutation rate in the bacteria cells, and reducing the frequency of the development of antibiotic resistance.

Colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be applied as an adjunct therapy to the administration of one or more chemotherapeutic agents in any application in which an inhibitor of mutagenesis and/or carcinogenesis is desired, preventing the development of multi-drug resistant tumor cells due to additional genomic mutations, and lower formation of tumor cells responsible for metastasis.

Colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, may be applied as an adjunct therapy to the administration of one or more chemotherapeutic agent(s) in any application in which an inhibitor of mutagenesis and/or carcinogenesis is desired, preventing the development of mutations in normal cells caused by radio- and chemotherapeutic agents.

The present invention also provides a composition that includes one or more active agents (i.e., colostrinin, at least one constituent peptide thereof, or active analog thereof) of the invention and one or more carriers, preferably a pharmaceutically acceptable carrier. The methods of the invention include administering to, or applying to the skin of, a patient, preferably a mammal, and more preferably a human, a composition of the invention in an amount effective to produce the desired effect. The active agents of the present invention are formulated for enteral administration (oral, rectal, etc.) or parenteral administration (injection, internal pump, etc.). The administration can be via direct injection into tissue, interarterial injection, intervenous injection, or other internal administration procedures, such as through the use of an implanted pump, or via contacting the composition with a mucus membrane in a carrier designed to facilitate transmission of the composition across the mucus membrane such as a suppository, eye drops, inhaler, or other similar administration method or via oral administration in the form

of a syrup, a liquid, a pill, capsule, gel coated tablet, or other similar oral administration method. The active agents can be incorporated into an adhesive plaster, a patch, a gum, and the like, or it can be encapsulated or incorporated into a bio-erodible matrix for controlled release.

The carriers for internal administration can be any carriers commonly used to facilitate the internal administration of compositions such as plasma, sterile saline solution, IV solutions or the like. Carriers for administration through mucus membranes can be any well-known in the art. Carriers for administration oral can be any carrier well-known in the art.

The formulations may be conveniently presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Methods may include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

Formulations suitable for parenteral administration conveniently include a sterile aqueous preparation of the active agent, or dispersions of sterile powders of the active agent, which are preferably isotonic with the blood of the recipient. Isotonic agents that can be included in the liquid preparation include sugars, buffers, and sodium chloride. Solutions of the active agent can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions of the active agent can be prepared in water, ethanol, a polyol (such as glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, glycerol esters, and mixtures thereof. The ultimate dosage form is sterile, fluid, and stable under the conditions of manufacture and storage. The necessary fluidity can be achieved, for example, by using liposomes, by employing the appropriate particle size in the case of dispersions, or by using surfactants. Sterilization of a liquid preparation can be achieved by any convenient method that preserves the bioactivity of the active agent, preferably by filter sterilization. Preferred methods for preparing powders include vacuum drying and freeze drying of the sterile injectible solutions. Subsequent microbial contamination can be prevented using various antimicrobial agents, for example, antibacterial, antiviral and antifungal agents including parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Absorption of the active

agents over a prolonged period can be achieved by including agents for delaying, for example, aluminum monostearate and gelatin.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as tablets, troches, capsules, lozenges, wafers, or cachets, each containing a predetermined amount of the active agent as a powder or granules, as liposomes containing the active agent, or as a solution or suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, or a draught. The amount of active agent is such that the dosage level will be effective to produce the desired result in the subject.

Nasal spray formulations include purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes. Formulations for rectal or vaginal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids. Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye. Topical formulations include the active agent dissolved or suspended in one or more media such as mineral oil, DMSO, polyhydroxy alcohols, or other bases used for topical pharmaceutical formulations.

Useful dosages of the active agents can be determined by comparing their *in vitro* activity and the *in vivo* activity in animal models. Methods for extrapolation of effective dosages in mice, and other animals, to humans are known in the art; for example, see U.S. Patent No. 4,938,949.

The tablets, troches, pills, capsules, and the like may also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, fructose, lactose or aspartame; and a natural or artificial flavoring agent. When the unit dosage form is a capsule, it may further contain a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac, or sugar and the like. A syrup or elixir may contain one or more of a sweetening agent, a preservative such as methyl- or

propylparaben, an agent to retard crystallization of the sugar, an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol, a dye, and flavoring agent. The material used in preparing any unit dosage form is substantially nontoxic in the amounts employed. The active agent may be incorporated into sustained-release preparations and devices.

Active agents can be administered alone or in various combinations to a patient (e.g., animals including humans) as a medication or dietary (e.g., nutrient) supplement in a dose sufficient to produce the desired effect throughout the patient's body, in a specific tissue site, or in a collection of tissues (organs).

As used herein, "a" or "an" means one or more (or at least one), such that combinations of active agents (i.e., active oxidative stress regulators), for example, can be used in the compositions and methods of the invention. Thus, a composition that includes "a" polypeptide refers to a composition that includes one or more polypeptides.

"Amino acid" is used herein to refer to a chemical compound with the general formula: NH_2---CRH---COOH, where R, the side chain, is H or an organic group. Where R is organic, R can vary and is either polar or nonpolar (i.e., hydrophobic). The amino acids of this invention can be naturally occurring or synthetic (often referred to as nonproteinogenic). As used herein, an organic group is a hydrocarbon group that is classified as an aliphatic group, a cyclic group or combination of aliphatic and cyclic groups. The term "aliphatic group" means a saturated or unsaturated linear or branched hydrocarbon group. This term is used to encompass alkyl, alkenyl, and alkynyl groups, for example. The term "cyclic group" means a closed ring hydrocarbon group that is classified as an alicyclic group, aromatic group, or heterocyclic group. The term "alicyclic group" means a cyclic hydrocarbon group having properties resembling those of aliphatic groups. The term "aromatic group" refers to mono- or polycyclic aromatic hydrocarbon groups. As used herein, an organic group can be substituted or unsubstituted.

The terms "polypeptide" and "peptide" are used interchangeably herein to refer to a polymer of amino acids. These terms do not connote a specific length of a polymer of amino acids. Thus, for example, the terms oligopeptide, protein, and enzyme are included within the definition of polypeptide or peptide, whether produced using recombinant techniques, chemical or enzymatic synthesis, or

naturally occurring. This term also includes polypeptides that have been modified or derivatized, such as by glycosylation, acetylation, phosphorylation, and the like.

The following abbreviations are used throughout the application:

A = Ala = Alanine	T = Thr = Threonine
V = Val = Valine	C = Cys = Cysteine
L = Leu = Leucine	Y = Tyr = Tyrosine
I = Ile = Isoleucine	N = Asn = Asparagine
P = Pro = Proline	Q = Gln = Glutamine
F = Phe = Phenylalanine	D = Asp = Aspartic Acid
W = Trp = Tryptophan	E = Glu = Glutamic Acid
M = Met = Methionine	K = Lys = Lysine
G = Gly = Glycine	R = Arg = Arginine
S = Ser = Serine	H = His = Histidine

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1

Colostrinin decreases spontaneous and induced mutation frequencies at the hprt locus in Chinese hamster V79 cells

Colostrinin (CLN), a uniform mixture of low-molecular weight, proline-rich polypeptides, induces neurite outgrowth of pheochromocytoma cells, extends the lifespan of diploid fibroblast cells, inhibits beta amyloid-induced apoptosis and resulted in improved cognitive function when administered to Alzheimer's patients. The present example investigated CLN's antimutagenic activity in cells stressed oxidatively or exposed to chemical or physical agents and shows that CLN did not alter cell cycle kinetics and cloning efficiency, while it inhibited the development of spontaneous mutations at the coding region of the hypoxanthine phosphoribosyl-transferase (*hprt*) gene in Chinese hamster V79 cells. In a dose-dependent manner, CLN lowered reactive oxygen species (ROS)-induced frequency of cells resistant to 6-thioguanine (6-TG) to nearly background level. Likewise, CLN decreased the frequency of methyl methanesulfonate- or mitomycin C-induced mutations in V79 cells. Notably, CLN (at 100, 250, and 500 ng per ml concentrations) decreased UVA-induced mutation frequency. The highest dose of CLN also decreased significantly the number of UVB-induced 6-TG-resistant mutant cells. Similar results were obtained using cell cultures of human origin. Overall, this example shows that CLN significantly lowers the mutation frequency that develops spontaneously or is induced by ROS, chemical and physical agents. CLN itself has no mutagenic activity. Therefore, CLN may be used in human therapies systemically and/or locally for the prevention of diseases associated with sequence alterations in genomic and mitochondrial DNA.

CLN is being used in both animal studies and clinical trials. Considering CLN's possible future use in systemic and local applications, it is important to define its toxicity and mutagenicity. The capacity of CLN to prevent/protect cells from a mutagenic effect of reactive oxygen species (ROS) and physical and chemical agents such as ROS generated by glucose oxidase (GOx) (Das et al. *J Biol Chem* 280: 35272-35280, 2005) UVA, UVB, methyl methanesulfonate (MMS), and mitomicyn C (MMC) was studied. UVA primarily induces ROS in the presence of

endogenous photosensitizers. UVB is absorbed by DNA that leads to direct DNA damage (Pfeifer et al. *Mutat Res* 571: 19-31, 2005). MMS is a monofunctional DNA alkylating agent (Bartsch et al. *Mutat Res* 110: 181-219, 1983). MMC produces inter- or intra-strand DNA cross-links, single- and double-strand breaks (Waring. *Annu Rev Biochem* 50: 159-192, 1981), and also triggers ROS generation in mammalian cells (Waring. *Annu Rev Biochem* 50: 159-192, 1981). To evaluate toxic and antimutagenic characteristics of CLN, we used hamster lung fibroblast (V79) cell cultures. V79 cell cultures have been used since 1970 to study the mutagenic and clastogenic potential of various man-made and environmental agents (reviewed in Bradley et al. *Mutat Res* 87: 81-142, 1981). Data were confirmed using human bronchial epithelial cells.

This example shows, for the first time, that treatment of cultured mammalian cells with CLN induced neither mutation nor a mediated toxic effect, but rather that CLN significantly decreased spontaneous and ROS-induced mutation frequency. Its antimutagenic effect was also significant when cells were exposed to UV or chemical agents. These properties of CLN make it very attractive natural substance for the prevention and/or treatment of human diseases.

Materials and Methods

Cell Cultures. V79 cells (American Type Culture Collection (ATCC), Rockville, MD, USA) were maintained in Eagle's minimum essential medium (EMEM; Invitrogen) containing 10% fetal bovine serum (FBS), supplemented with glutamine (292mg/L), streptomycin (100 μg/ml), and penicillin (100 U/ml) as previously described (Albrecht et al. *Virology* 230: 48-61, 1997). Prior to the mutation studies, the cells were cultured in HAT medium containing 5×10^{-5} M hypoxanthine (Hyp), 4×10^{-7} M aminopterin, 5×10^{-6} M thymidine, and stored in numerous replicate vials in liquid nitrogen. A549 bronchial epithelial cells were purchased from ATCC, and cultured in F-12 Kaighn's-modified medium. The cell cultures were routinely examined for mycoplasma contamination. Population doubling (PD) levels were calculated as follows: At 90 percent confluence, cells were removed by trypsinization, and counted, and population doublings were calculated (PD = log(number of cells obtained at subculture per 10^5)/$\log_2$) (Szczesny et al. *Proc Natl Acad Sci U S A* 100: 10670-10675, 2003).

Mutational Analysis. V79 cells were recovered from liquid nitrogen, cultured in growth medium, containing HAT medium until 90% confluency was

attained. The cells were reseeded at a density of 5 x 10^5 cells per dish in growth medium, allowed to proliferate overnight, and then were treated with oxidative stress-inducing agents or exposed to chemical or physical agents. Cells were washed three times with PBS, and 24 hours later monolayers were dissociated and subcultured into 100-mm dishes (5x10^5 cells/ dish) in growth medium containing 6-thioguanine (6-TG; 7 μg/ml) to identify presumptive mutants or in nonselective medium to measure the plating efficiency. Colonies of 6-TG-resistant cells were allowed to develop for 6 to 8 days, fixed in 3.7% formalin, and stained with 0.1% crystal violet. The mutation frequency was calculated by determining the number of 6-TG-resistant colonies relative to the number of cells seeded in the selective medium, and normalized for the plating efficiency in the absence of the selective agent (Bradley et al. *Mutat Res* 87: 81-142, 1981; Albrecht et al., *Virology*, 230:48-61, 1997).

Determination of HPRT Activity. Incorporation of hypoxanthine into mutant and wild-type cells was evaluated as previously described (Albrecht et al. *Virology* 230: 48-61, 1997). Briefly, wild-type and 6-TG-resistant cell cultures at 75% confluency were incubated in growth medium, containing 5 x 10^{-6} M [^{3}H]hypoxanthine (specific 750 mCi/mmol). Following lysis of the cells with 1 ml of lysis solution (1% SDS, 2% Na_2CO_3, 0.1 N NaOH), the lysate was combined with an equal volume of ice-cold 20% trichloroacetic acid. The resulting precipitate was collected by filtration onto Whatman grade 3 filter disks, and the radioactivity was determined by liquid scintillation spectrophotometry.

Clonogenic Survival Assay. V79 cells were subcultured at a density of 200 cells per 60-mm Petri-dish. Four hours later, 4 ml of fresh medium was added to parallel cultures containing increasing doses of the test substance. CLN was present for 24 hours, while MMS and GOx treatment was for 1 hour. To determine the effect of UV-irradiation, the medium of the cell culture was removed, and cells were exposed to increasing UV doses. After treatment cells were fed with fresh medium and incubated further for 7 to 10 days. When colonies reached 1 mm in diameter, cells were fixed with 3.7% formalin (30 min) and stained with crystal violet. Colonies containing 20 or more cells were counted. LD_{10} values were defined as the concentration of agent resulting in 10% inhibition of colony formation. Accurate dose values were estimated from nonlinear regression analysis of concentration–response curves (Boldogh et al. *Cancer Res* 58: 3950-3956, 1998).

Treatment of Cells. Cell cultures at appropriate confluency were treated with GOx (20 ng per ml), MMS (115 μM) or MMC (25 μM) in serum-free medium for 1 hour. At the end of the treatment, cells were washed with PBS to remove the excess toxic compounds and placed under growth medium. CLN at the indicated concentrations was added to the culture media 24 hours prior and 24 hours after treatment with genotoxic agents. In selected experiments, CLN was added either prior to or after treatment.

Irradiation of Cells. Cell cultures (10 cm Petri-dishes) at 50% confluency were irradiated for 24 hours, and then seeded into selection medium. The source of UV-irradiation was UVP, 8W Model UVLMS-38 (Upland CA). The UV doses used in these studies were 4.1 kJ/m^2 for UVB (302 nm) and 87 kJ/m^2 for UVA (365 nm). UVA irradiation was carried out in the presence of the photosensitizer riboflavin (100 μg per ml). The UV doses were determined with a UVX Digital Radiometer, UVP (Upland, CA) before each treatment.

Cell Cycle Analysis. Cell cycle analyses were carried out as previously described (Bresnahan et al. *Virology* 231: 239-247, 1997). Briefly, cells were washed in PBS, and collected by sedimentation. Cells were incubated in low salt buffer (3% polyethylene glycol, 5 mg/ml propidium iodine, 0.1% Triton X-100, 4 mM sodium citrate, 100 μg/ml RNase A) for 20 minutes at 37°C. High salt buffer (3% polyethylene glycol, 5 μg/ml propidium iodine, 0.1% Triton X-100, 400 mM NaCl) was added. The cell cycle stage was determined via cellular DNA content, which was analyzed using a Becton–Dickinson FACScan flow cytometer.

Measurement of Intracellular ROS Level. Changes in intracellular ROS levels were determined as described previously (Boldogh et al. *Toxicology* 193: 137-152, 2003). Briefly, the cells were suspended in PBS and incubated with 5 μM 5(and 6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes) for 15 minutes at 37°C. The changes in DCF fluorescence were determined by flow cytometry. The mean fluorescence for 12,000 cells from three or more independent experiments was analyzed, and results are expressed as ±SEM.

Annexin V Assay. To show exposure of phosphatidyl serine on the outer side of the cytoplasmic membrane, flow cytometric analyses were performed on cells stained with annexin V/PE and 7-amino actinomycin D (7-AAD) (4). Briefly, cells were grown to 70% confluency on 60-mm Petri dishes, and treated with CLN for 24-48 hours at 37°C. After treatment, cells were trypsinized, washed in PBS, and

resuspended in cold Annexin V binding buffer (10 mM HEPES, pH 7.4, 0.14 mM NaCl, 0.25 mM $CaCl_2 \cdot 2H_2O$, 0.1% BSA). Annexin-PE (BD Biosciences) was added to the cells for 30 minutes, and 7-AAD (2 μg/ml) was then added before analysis in a FACScan flow cytometer (Becton Dickinson FACscan). The mean fluorescence for 12,000 cells from three or more independent experiments was determined.

Statistical Analysis. The data were statistically analyzed by one-way ANOVA followed by Tukey's test for multiple pair-wise comparisons in all the test systems. Results were considered significant at $p<0.05$.

Results

CLN decreases spontaneous mutation frequency and shows no toxicity. To determine the effect of CLN on mutation frequency (MF) at the *hprt* locus, V79 cells were treated with increasing doses (10, 25, 50, 100, 250, 500, 2500 and 5000 ng per ml) of CLN for 48 hours, and cells were reseeded in selection medium containing 6-TG. Means for the quantitative data from three independent experiments and the relationship between the CLN concentration and the calculated MF are plotted in Fig. 1A. CLN at 10, 25 and 50 ng per ml CLN did not alter frequencies, while 100, 250, 500 and 2500 ng per ml CLN decreased spontaneous MF as shown in Fig. 1A. The highest concentration (5000 ng/ml) lowered, but did not significantly change MF. In a control experiment, cells were treated with glucose oxidase (GOx; 20 ng per ml).

Next, whether long-term treatment with CLN changes the spontaneous mutation frequency was investigated. V79 cells were maintained in the presence of CLN for approximately 40 population doublings, which equals 11 passages. Results are summarized in Fig. 1B. In the controls, due to extensive subculturing-induced stress, the spontaneous MF of V79 cells increased from $1.31 \pm 0.23 \times 10^{-6}$ to $4.72 \pm 0.43 \times 10^{-6}$. Remarkably, when cells were maintained in media containing 25, 50, 100, 250, 500, 2500 and 5000 ng per ml CLN, no increase in spontaneous MF was observed. These results suggest that CLN mediated a genomic stability and decreased exogenously generated stress levels due to the sub-culturing of cells (Fig. 1B).

Next, cells were grown in the presence of CLN (100, 250, 500, 2500 and 5000 ng per ml) for 4.4 PDs, and cell cycle stage distribution, plating efficiency, and

toxicity were determined. None of the concentrations of CLN showed any change in the percentage of cells in the S-phase of the cell cycle (cell proliferation) or plating efficiency. Furthermore, there was no indication of toxicity (phosphatidyl-serine exposure on the outer side of the cytoplasmic membrane) demonstrated by Annexin V assays (Table 1).

Table 1

CLN has no Effect on Cell Proliferation, Plating Efficiency and Frequency of Annexin-V Binding Cells

CLN (ng per ml)	[a]Percentage of cells in S-phase	[b]Percent of Annexin V Positive Cells	[c]Plating Efficiency
0	6.7	0.12	95.4
100	8.3	0.11	96.7
250	7.7	0.045	96.1
500	8.1	0.05	95.9
2500	7.95	0.1	95.3
5000	7.92	0.08	96.4

Data shown are the average of three independently undertaken experiments in duplicate. [a]The cellular DNA content and [b] the annexin V staining was determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson). Twelve thousand events were collected from each sample. [c]Plating efficiencies were determined as described in Materials and Methods.

Antimutagenic activity of CLN in oxidatively stressed cells. In the next series of experiments, whether CLN prevents ROS-induced mutagenicity was explored. GOx was used as a source of exogenously generated ROS, which produces DNA-damaging radicals during a free radical cascade (Das et al. *J Biol Chem* 280: 35272-35280, 2005). First, the cytotoxic effect of increasing concentrations of GOx was examined in clonogenic survival assays. When cells were treated at ~30% confluence for 1 hour with 40, 30, 20 and 10 ng per ml GOx, plating efficiencies were 53, 76, 88, and 95.6 %, respectively. Measurement of

intracellular levels of ROS by DCF assays showed that oxidative stress levels were increased by 3.5-, 3.2-, 2.2-, and 1.45-fold after 40, 30, 20 and 10 ng per ml GOx addition, respectively. For further studies, 20 ng per ml GOx was used. Parallel cultures of cells at low density (~30 % confluence) were mock-treated or treated with 25, 50, 100, 250, 500, 2500 and 5000 ng per ml CLN for 24 hours and exposed to 20 ng per ml GOx for 1 hour. Cells were washed with PBS to remove GOx, and CLN was added in the culture medium for 24 hours, and then cells were reseeded in 6-TG containing selection medium. GOx alone increased MF from 1.47 $x10^{-6}$ to 30.7 $x10^{-6}$. Results summarized in Fig. 2A show that CLN decreased GOx-induced MF in a dose-dependent manner. For example, 100, 250 and 500 ng per ml CLN decreased MF from 30.7 $x10^{-6}$ to 6.7 $x10^{-6}$, 2.6 $x10^{-6}$ and 2.5 $x10^{-6}$, respectively (Fig. 2A).

Next, whether pre- or post-treatment of cells with CLN decreases ROS-induced mutation frequency was tested. Cells were grown in the presence of CLN for 24 hours and GOx-treated for 1hour. GOx was removed by washing, and 24 hours later cells were reseeded in selection media. The result showed that pre-treatment of cells with CLN was sufficient to decrease MF. CLN at 100, 250 and 500 ng per ml concentration decreased MF from 34.7 $x10^{-6}$ to 19.8 $x10^{-6}$, 17.4 $x10^{-6}$ and 12.9 $x10^{-6}$, respectively (Fig. 2B). Next, cells were grown in CLN-free medium and GOx-treated for one hour. After washing the cells CLN was added. CLN at 100, 250 and 500 ng per ml concentration significantly lowered MF from 28.7 $x10^{-6}$ to 20.8 $x10^{-6}$, 7.9 $x10^{-6}$ and 7.1 $x10^{-6}$, respectively (Fig. 2C). There was no HPRT activity detectable in 6-TG resistant clones. These data together show, that CLN is effective in controlling the mutation frequency induced by ROS.

Effect of CLN on UVA- and UVB-induced mutation frequency. To determine further whether CLN is effective against ROS-induced genomic instability, cells were irradiated with UVA or UVB. Cellular toxicity of UV radiation was determined in clonogenic survival assays. To test CLN's antimutagenic effect, LD_{10} doses of UVA and UVB irradiation were used. Accordingly, parallel cell cultures at ~50% confluence were irradiated with 87 kJ per m^2 UVA or 4.1 kJ per m^2 UVB, and 24 hours later cells were reseeded in selection medium containing 6-TG (7 μg per ml). UVB irradiation induced a higher number of 6-TG resistant cell clones (96±14 x 10^{-6} cells), while UVA radiation was less mutagenic (19.7 x 10^{-6}). The results are in accordance with data published

previously for V79 cells (Dahle and Kvam, *Cancer Res* 63: 1464-1469, 2003, Wells and Han, *Mutat Res* 129: 251-258, 1984).

To test the effect of CLN, cells were grown in CLN (at 100, 250, and 500 ng per ml)-containing medium for 24 hours, UV-irradiated, and further incubated in the presence of CLN (100, 250, and 500 ng per ml) for 24 hours. As above, 6-TG was added, cells formed, survival colonies counted and mutation frequencies were calculated. As summarized in Figs. 3A and 3B, CLN decreased UVA-induced MF significantly in a concentration-dependent manner. MF induced by UVB was substantially decreased (100 and 250 ng/ml); however, at the highest CLN concentration (500 ng per ml) its effect was significant. Randomly selected 6-TG-resistant clones showed no HPRT activity. These data support that CLN protects not only against oxidative stress-induced genotoxic insults, but also decreases UVB-induced mutagenesis, which implies the action of other type(s) of protective mechanism(s).

CLN decreases chemical agent-induced mutation frequency. Next, the efficacy of CLN in controlling the MF induced by MMS, a monoalkylating agent, was determined. The LD_{10} of MMS was 115 μM in clonogenic survival assays. Cells at ~30% confluence were incubated with CLN (100, 250, 500 ng per ml) for 24 hours followed by MMS (115 μM) for 1 hour. MMS was removed by washing and the same amount of CLN was added to the corresponding cultures. In controls, MMS at 115 μM induced 28.6±3.6 mutants per 10^6 cells at *hprt* locus. At 100 and 250 ng per ml CLN substantially lowered, while 500 ng per ml of CLN significantly decreased MMS-induced MF as shown in Fig. 4A.

MMC is extensively used for treatment of diseases, such as malignancies (Bradner et al. *Cancer Treat Rev* 27: 35-50, 2001) although its mutagenicity is well documented (Davies et al. *Mutat Res* 291: 117-124, 1993). Therefore, whether CLN would decrease MMC-induced MF was tested. Its LD_{10} was 25 μM, as determined in preliminary studies. At this concentration, MMC caused an MF of 58.2±5.5 x 10^{-6} cells. When cells were pre- and post-treated with CLN (as in MMS experiments), the number of mutant colonies decreased significantly. At 100 ng per ml CLN did not decrease the number of 6-TG-resistant clones, while 250 and 500 ng per ml decreased them by 39 and 58 %, respectively (Fig. 4B). Randomly selected 6-TG-resistant clones from MMS and MMC experiments showed no detectable HPRT activity.

CLN decreases mutation frequency in human cells. Selected experiments were carried out using A549 human airway epithelial cells. Parallel cultures of cells were treated with CLN (250, 500 ng per ml) for 24 hours, and then exposed to LD_{10} (determined in preliminary studies) of GOx (110 ng per ml for 1 hour); UVA (105 kJ per m^2), UVB 7.1 kJ per m^2) and MMC (53 μM for 1 hour). Cells were washed, and incubated with CLN-containing growth medium for 52 hours (two PDs). Cells were reseeded in Petri dishes (1 x 10^6 cells per 10 cm dish) in 6-TG containing media. Mutation frequencies were calculated and results are shown in Fig. 5. Although, the LD_{10} dose of these agents for A549 cells was higher than that for V79 cells, the overall results were similar. Namely, CLN significantly decreased MF induced by ROS (GOx, UVA) and the DNA-alkylating agent MMC. CLN less effectively, but significantly, lowered the UVB-induced mutation frequency. Randomly selected 6-TG resistant clones possessed no HPRT activity. These data together suggest that CLN decreases MF in cells of rodent and human origin. These data are also as published in Basci et al., (*J Exp. Ther. Oncol* 5:249-259, 2006).

Discussion

CLN, composed of low-molecular weight, proline-rich polypeptides, induces maturation and differentiation of murine thymocytes, production of cytokines, and neurite outgrowth of pheochromocytoma cells, decreases oxidative stress-mediated activation of c-Jun NH2-terminal kinases, and inhibits beta amyloid-induced apoptosis in cultured cells (Janusz et al. *Arch Immunol Ther Exp (Warsz)* 41: 275-279, 1993, Bacsi et al. *Cell Mol Neurobiol* 25: 1123-1139, 2005, Stanton et al. *Psychogeriat. Annuals* 4: 67-75, 2001, Boldogh et al. *Psychogeriat. Annuals* 4: 57-65, 2001, Schuster et al. *Neuropeptides* 39: 419-426, 2005). The present study sought to determine the toxicity and antimutagenic potential of the CLN treatment. This example shows that CLN had no effect on cell proliferation, and plating efficiency, and did not induce the expression of phosphatidyl serine on the outer cytoplasmic membrane, considered an early sign of cellular toxicity. Moreover, this example demonstrates that CLN decreases the frequency of mutations developing spontaneously, decreases the frequency of mutations induced by ROS, decreases the frequency of mutations induced by chemical agents, decreases the frequency of mutations induced by UVA, and decreases the frequency of mutations induced by UVB. These data underline its potential for wide-ranging human applications.

Oxidative stress is caused by an imbalance between the ROS-generating and -eliminating mechanisms of cells (Lander et al. *Nature* 381: 380-381, 1996). Oxidatively damaged lipids and proteins are eliminated by degradation, while DNA is repaired (Mitra et al. *Free Radic Biol Med* 33: 15-28, 2002). Mutations in genes occur when oxidatively and/or chemically modified bases, abasic sites, and DNA strand breaks are not repaired correctly. Modifications in DNA sequence order may result in proteins that possess an altered structure and/or activity leading to dysregulation of the cellular redox balance, alteration in cell activation cascades, cell cycle, and manifestation of patho-biological processes causing diseases (Mitra et al. *Free Radic Biol Med* 33: 15-28, 2002). Importantly, ROS-, chemical- and physical agents-mediated DNA sequence alterations are implicated in multistage carcinogenesis (Hanahan et al. *Cell* 100: 57-70, 2000).

Because oxidative damage to DNA is the most common, the antimutagenic effect of CLN in oxidatively stressed cells was first tested. Oxidative stress was produced by GOx, a known ROS generator (Nilsson et al. *Biochim Biophys Acta* 192: 145-148, 1969). This approach provided more consistent results than challenging cells with H_2O_2 (Das et al. *J Biol Chem* 280: 35272-35280, 2005). The data show that when CLN was present prior to, during and after oxidative insult of the cells, MF was decreased to nearly background level. Notably, CLN also decreased mutation frequency when it was added prior to or after exposure to ROS. At present, the mechanism by which CLN exerts its antimutagenic activity in oxidatively stressed cells is unclear. It is possible that CLN increases the activity of antioxidant enzymes and/or levels of cellular low-molecular weight antioxidants, such as glutathione, which thereby decreases the levels of exogenously introduced ROS, consequently resulting in oxidative damage to DNA. Another possibility is that CLN, by regulating cellular redox, increases the efficacy of the DNA base excision repair (BER) machineries, the major pathways repairing oxidatively damaged DNA (Pines et al. *Nucleic Acids Res* 33: 4379-4394, 2005, Szczesny et al. *Mech Ageing Dev* 125: 755-765, 2004). For example, there is evidence showing the importance of redox-dependent, posttranslational modifications of APE1/ref-1, a rate-limiting protein in BER. These modifications of APE1 are critical for interaction with members of the repair machinery and its endonuclease activity (Ramana et al. *Proc Natl Acad Sci U S A* 95: 5061-5066, 1998, Kelley et al. *Antioxid Redox Signal* 3: 671-683, 2001).

To test further whether CLN is effective only in suppressing ROS-induced mutagenesis, cells were irradiated with UVA or UVB. UVA radiation-induced genomic instability has been linked to production of ROS, while UVB is absorbed by DNA leading to direct DNA damage (Wells et al. *Mutat Res* 129: 251-258, 1984, Hockberger et al. *Proc Natl Acad Sci U S A* 96: 6255-6260, 1999). At concentrations 100 ng per ml or higher, CLN significantly inhibited UVA-induced immediate mutagenesis, while only the highest (500 ng per ml) CLN concentration decreased significantly the MF induced by UVB. Taken together these data suggest that the antimutagenic properties of CLN are partly due to its effect on cellular redox balance and also to its possible impact on repair of UVB-induced DNA damage. The later possibility is presently being investigated. Also, we have no data showing whether CLN alters levels of DNA damage, especially during UVB radiation. Regardless of the mechanism, these findings have great significance, because UVB is the primary cause of squamous cell carcinomas (de Gruijl et al. *Cancer Res* 53: 53-60, 1993), and UVA irradiation plays a role in induction of malignant melanoma (Setlow et al. *Proc Natl Acad Sci U S A* 90: 6666-6670, 1993, Ley, *Cancer Res* 57: 3682-3684, 1997). The UV doses chosen for the experiments in this example are physiologically relevant because they were equivalent to midday sun exposure for 30 and 100 min for UVA and UVB, respectively (Kolari et al. *Photodermatol* 3: 340-345, 1986, Meloni et al. *Photochem Photobiol* 71: 681-690, 2000). These data imply that CLN would likely be suitable for human application to decrease UVA- and UVB-induced cellular damage, and thereby their well-known carcinogenic effects (Setlow et al. *Proc Natl Acad Sci U S A* 90: 6666-6670, 1993, Ley, *Cancer Res* 57: 3682-3684, 1997).

CLN significantly decreased MF induced by DNA-damaging chemical agents. For these studies, MMS and MMC were selected. MMS, a DNA mono-alkylating agent, induced a relatively low MF at the *hprt* locus of V79 cells, which is in line with previous reports (Nishi et al. *Cancer Res* 44: 3270-3279, 1984, Yamada et al. *Mutat Res* 471: 29-36, 2000). MMS primarily alkylates guanine at the O^6-position, which is repaired by O^6-methylguanine DNA methyltransferase (MGMT) (Margison et al. *Carcinogenesis* 24: 625-635, 2003). At lower concentrations (100, 250 ng/ml), CLN substantially decreased MMS-induced MF, but did so significantly at higher concentration (500 ng/ml). Because MMS-induced genomic mutations are related primarily to guanine alkylation, and MGMT's action is not redox regulated,

these results suggest that CLN decreases MF in a manner independent from its impact on cellular redox status in MMS-treated cells.

MMC is a DNA cross-linking alkylating agent that, after getting through the cell membrane, is activated by NAD(P)H quinone oxido-reductase, xanthine dehydrogenase, xanthine oxidase, cytochrome *p*450 reductase and/or cytochrome b_5 reductase (Collier et al. *Life Sci* 78: 1499-1507, 2006). By directly interacting with DNA, MMC induces an array of damage, including DNA inter- and intra-crosslinks, single-strand breaks and double-strand breaks. MMC treatment also increases ROS levels and consequently, oxidative damage to DNA. Taking into consideration that CLN decreases MF in ROS-, and UVB- as well as MMS-treated cells, the significant decrease in MMC-induced MF by CLN is not surprising. Further experiments are in progress to define the utility of CLN in decreasing the side effects of MMC with respect to lower ROS levels and/or its mutagenicity.

Taken together, these results suggest that the antimutagenic properties of CLN are provided by multiple mechanisms. It may exert its antimutagenic action by decreasing intracellular ROS levels, and thereby preventing DNA damage. Alternatively, CLN may increase the efficacy of DNA repair via generation of favorable cell activation signals and/or cellular response to DNA damage. These findings are highly significant because natural compounds that prevent the genotoxic effects of endogenous and/or environmental mutagens have great importance (Odin, *Mutat Res* 386: 39-67, 1997).

Mutational analysis has been an important approach for testing the genotoxicity of biologically active natural compounds such as CLN. This example shows that CLN is potent in decreasing mutation frequency induced spontaneously, decreasing mutation frequency induced ROS, decreasing mutation frequency induced UV radiation, and decreasing mutation frequency induced mono- and bi-functional alkylating agents. These results suggest that CLN is capable of protecting cells from genomic instability in two different, but overlapping mechanisms: 1) decreasing the cellular levels of ROS and oxidative damage to DNA and/or 2) increasing the efficacy of major DNA repair pathway(s). These properties of CLN, a proline-rich mix of peptides, demonstrate the safety of CLN in human applications.

Example 2

Following the methods described in more detail in Example 1, one or more of the constituent peptides of colostrinin described herein, including, but not limited to MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIPN (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ (SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP (SEQ ID NO:24); QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26); LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); VESYVPLFP (SEQ ID NO:31); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and MHQPPQPLPPTVMFP (SEQ ID NO:34), RPKHPIKHQGLPQEVLNENLLRF (SEQ ID NO:35), FVAPFPEVFGKEKV (SEQ ID NO:36), SDIPNPIGSENSEKTTMPLW (SEQ ID NO:37), GPVRGPFPI (SEQ ID NO:38), EPVLGPVRGPFPI (SEQ ID NO:39), VPYPQRDMPIQ (SEQ ID NO:40), SLSQSKVLPVPQKAVPYPQRDMPIQ (SEQ ID NO:41), RPKHPIKHQ (SEQ ID NO:42); EPVLGPVR (SEQ ID NO:43); RGPFPIIV (SEQ ID NO:44); PPPPLLPSRPLPPLPPP (SEQ ID NO:45); PPPPPPPPP (SEQ ID NO:46), analogs thereof, and combinations thereof may be assayed for anti-mutagenic effects.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

SEQUENCE LISTING FREE TEXT

SEQ ID NO:1-46 Colostrinin constituent peptides

What is claimed is:

1. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof.

2. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, under conditions effective to accomplish at least one of the following:

decrease the frequency of reactive oxygen-species (ROS) induced mutations;

decrease the frequency of mutations induced by chemical agents; or

decrease the frequency of mutations induced by physical agents.

3. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by chemical agents and/or physical agents and is not induced by reactive oxygen-species (ROS).

4. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced not only by reactive oxygen-species (ROS), but is also induced also by chemical agents and/or physical agents.

5. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is

induced by a chemical agent and/or a physical agent and is not induced by UVA or UVB radiation.

6. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is UVA radiation-induced mutagenesis.

7. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is UVB-radiation induced mutagenesis.

8. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by a chemical agent.

9. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the mutation frequency is induced by a physical agent.

10. A method of decreasing spontaneous mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof.

11. A method of decreasing mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a

constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof, wherein the mutation frequency is environmentally induced.

12. A method of decreasing the mutation frequency in a cell, the method comprising contacting the cell with a modulator selected from the group of colostrinin, a constituent peptide of colostrinin, an active analog of a constituent peptide of colostrinin, and combinations thereof; wherein the modulator is administered in conjunction with one or more therapeutic agents that induce mutations in genomic and/or mitochondrial DNA.

13. The method of claim 12, wherein the therapeutic agent is chemotherapy or radiation therapy for cancer.

14. The method of any one of claims 1-13, wherein the modulator is a constituent peptide of colostrinin or an analog thereof.

15. The method of claim anyone of claims 1-14, wherein the constituent peptide of colostrinin is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLQPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), VESYVPLFP (SEQ ID NO:31), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34),

RPKHPIKHQGLPQEVLNENLLRF (SEQ ID NO:35), FVAPFPEVFGKEKV (SEQ ID NO:36), SDIPNPIGSENSEKTTMPLW (SEQ ID NO:37), GPVRGPFPI (SEQ ID NO:38), EPVLGPVRGPFPI (SEQ ID NO:39), VPYPQRDMPIQ (SEQ ID NO:40), SLSQSKVLPVPQKAVPYPQRDMPIQ (SEQ ID NO:41), RPKHPIKHQ (SEQ ID NO:42); EPVLGPVR (SEQ ID NO:43); RGPFPIIV (SEQ ID NO:44); PPPPLLPSRPLPPLPPP (SEQ ID NO:45); PPPPPPPPP (SEQ ID NO:46); and combinations thereof.

16. The method of any one of claims 1-15, wherein an active analog of a constituent peptide of colostrinin comprises a peptide having an amino acid sequence with at least about 15 percent proline and having at least about 70 percent structural similarity to one or more constituent peptides of colostrinin.

17. The method of any one of claims 1-16, wherein the cell is present in a cell culture, a tissue, an organ, or an organism.

18. The method of any one of claims1-17, wherein the cell is a mammalian cell.

19. The method of any one of claims 1-18, wherein the cell is a human cell.

20. Use of a compound selected from the group of colostrinin, a constituent peptide thereof, an active analog thereof, and combinations thereof in the manufacture of a medicament for reducing the mutation frequency in a cell.

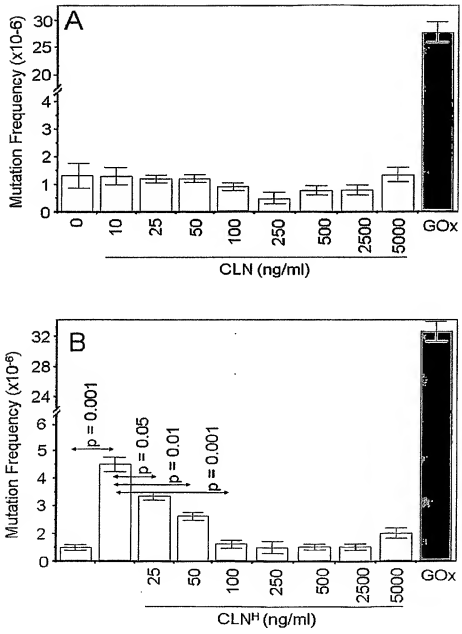
A
Mutation Frequency (x10-6)
30
25
20
4
3
2
1
0
0
10
25
50
100
250
500
2500
5000
GOx
CLN (ng/ml)
B
Mutation Frequency (x10-6)
32
28
24
6
5
4
3
2
0
p = 0.001
p = 0.05
p = 0.01
p = 0.001
25
50
100
250
500
2500
5000
GOx
CLNH (ng/ml)

Fig. 1

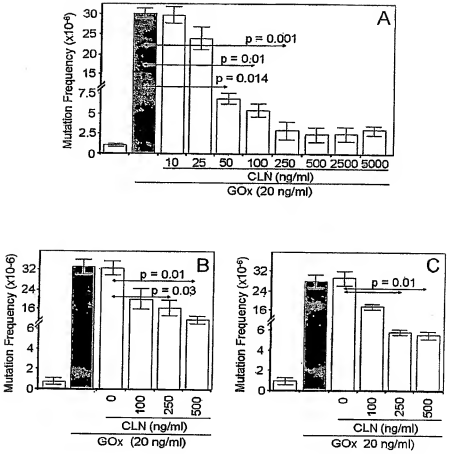
A
Mutation Frequency (x10-6)
30
25
20
15
7.5
5
2.5
0
p = 0.001
p = 0.01
p = 0.014
10 25 50 100 250 500 2500 5000
CLN (ng/ml)
GOx (20 ng/ml)
B
Mutation Frequency (x10-6)
32
24
16
6
4
2
0
p = 0.01
p = 0.03
0 100 250 500
CLN (ng/ml)
GOx (20 ng/ml)
C
Mutation Frequency (x10-6)
32
24
16
6
4
2
0
p = 0.01
0 100 250 500
CLN (ng/ml)
GOx 20 ng/ml)

Fig. 2

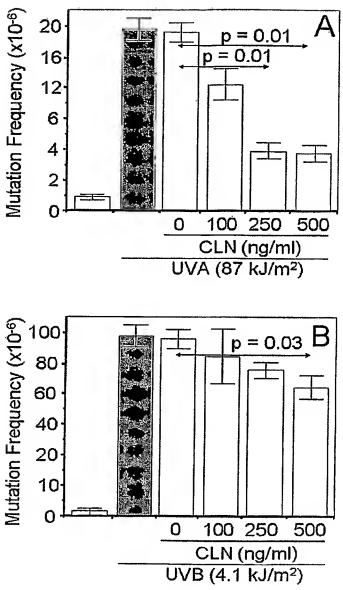
Mutation Frequency (x10-6)
20
16
12
6
4
2
0
p = 0.01
p = 0.01
A
0 100 250 500
CLN (ng/ml)
UVA (87 kJ/m2)
Mutation Frequency (x10-6)
100
80
60
40
20
10
0
p = 0.03
B
0 100 250 500
CLN (ng/ml)
UVB (4.1 kJ/m2)

Fig. 3

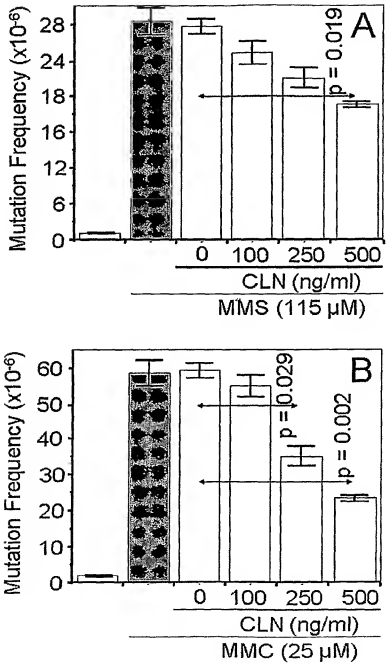
Mutation Frequency (x10-6)
28
24
18
16
12
6
0
A
p = 0.019
0 100 250 500
CLN (ng/ml)
MMS (115 µM)
Mutation Frequency (x10-6)
60
50
40
30
20
10
0
B
p = 0.029
p = 0.002
0 100 250 500
CLN (ng/ml)
MMC (25 µM)

Fig. 4

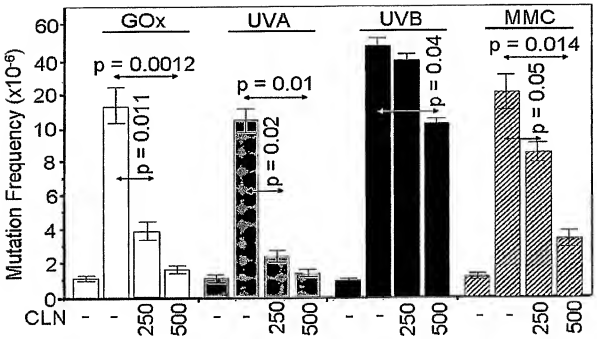
GOx
UVA
UVB
MMC
Mutation Frequency (x10-6)
60
40
20
10
8
6
4
2
0
p = 0.0012
p = 0.011
p = 0.01
p = 0.02
p = 0.04
p = 0.014
p = 0.05
CLN - - 250 500 - - 250 500 - - 250 500 - - 250 500

Fig. 5